NUCLEAR AND CYTOPLASMIC DNA SYNTHESIS IN ADULT AND EMBRYONIC ROTIFERS[1]

C. W. BIRKY, JR., ROSANNA ZITO BIGNAMI[2]
AND SISTER M. JACINTA BENTFELD

Department of Zoology, University of California, Berkeley, California 94720

During the embryonic development of rotifers and other Aschelminthes, most cells undergo a fixed number of mitotic divisions. Mitosis and cell division cease early in development; further differentiation thus leads to an adult with a fixed number of nuclei in each organ or tissue. Exceptions to this general rule, due presumably to variations in the number of nuclear divisions in certain organ primordia, are described by Birky and Field (1966). We have used autoradiography with tritiated thymidine to determine whether there is any DNA synthesis in the non-mitotic tissues of older embryos and adults of the rotifer *Asplanchna*. Our results indicate that nuclear DNA synthesis ceases when mitosis ceases, except in the vitellarium, a syncytial organ with 20 to 40 nuclei, analogous to the nurse cells of certain other organisms. In the vitellarium, nuclei are presumably becoming polyploid. We have also detected cytoplasmic DNA synthesis, apparently in mitochondria, in embryos and in adult vitellaria.

Materials and Methods

The studies were carried out on stocks of *Asplanchna brightwelli* from Indiana (inbred clones $5B4S_7 5$, $5B4S_5 3$, and $5B4S_6 3$; *cf.* Birky, 1967a) and Pennsylvania (clone 7B1-1; *cf.* Birky and Field, 1966). The results with the different stocks were identical, and will be considered together. Clones of amictic females, reproducing by diploid parthenogenesis, were reared on *Paramecium aurelia* in infusions of baked lettuce, Cerophyl, or Scottish grass at pH 7.5 and 23° as described by Birky (1964, 1967b).

Thymidine-methyl-H^3 was obtained from New England Nuclear Corporation (specific activity 6.7 c./m*M*) or Schwartz BioResearch Inc. (specific activity 6.0 c./m*M*). The final concentration of H^3-thymidine in the labelling medium was approximately 100 μc./ml. in all experiments. In some experiments adult females were exposed to H^3-thymidine in Dryl's (1959) or Gilbert's (1963) saline solution, for labelling of adult organs *in vivo* and female embryos *in utero*. In other experiments, embryos or adult tissues were dissected out into drops of pseudocoel cavity fluid under oil for labelling *in vitro*. H^3-thymidine in distilled water was then added to the drop; the technique is described in detail by Birky (1967b).

[1] Supported by USPHS research grant GM12183 to C. W. B. and by an NSF Institutional grant to the University of California.

[2] On leave of absence from: Laboratorio Radiobiologia Animale, Centro Studi Nucleari della Casaccia, CNEN, Roma, Italy.

Labelled tissues were processed in several different ways. (1) Adults, embryos, or vitellaria were fixed in 3:1 methanol:acetic acid, squashed in 45% acetic acid, and stained with aceto-orcein after hydrolysis for 10 minutes with 1 *N* HCl at 60°. (2) Adults were fixed in 3:1 ethanol:acetic acid, embedded in paraffin, and sectioned at 4 microns. (3) Adults, embryos, or vitellaria were fixed in glutaraldehyde (2.5% in Dryl's or Gilbert's) or OsO_4 (Palade's fluid, or 2.5% OsO_4 in 0.1 *M* phosphate buffer, pH 7.2–7.4) for one hour, embedded in Epon or methacrylate, and sectioned at $\frac{1}{2}$, 1, or 2 microns. In some cases, squashes or paraffin sections were treated in one of the following ways to test the specificity of the label: (1) deoxyribonuclease (DNase I, Worthington, RNase-free), 0.2 mg./ml. plus 0.003 *M* $MgSO_4 \cdot 7H_2O$ in Michaelis' veronal-acetate buffer without NaCl at pH 6.6 or in 0.01 *M* sodium phosphate buffer at pH 7.5, for 1, 3, or 6 hours at 37°; (2) ribonuclease (RNase, Worthington), 0.2 mg./ml. in Michaelis' buffer, pH 6.6, for 1, 3, or 6 hours at 37°; (3) trypsin (Worthington), 0.2 mg./ml. in 0.05 *M* sodium phosphate buffer, pH 7.4, 25 minutes at 37°. Control slides were treated for the same time and at the same temperature with the appropriate buffer or distilled water. Treated slides were exposd to ice-cold 5% trichloroacetic acid (TCA) for 20 minutes. After thorough washing in water, slides were covered with Kodak AR.10 stripping film or Ilford K.5 liquid emulsion (Caro, 1964) and stored with desiccant in light-tight boxes at 4°. Autoradiographs were usually developed after one to three weeks exposure. Paraffin sections were then stained with Harris hematoxylin; plastic sections were stained with 1% toluidine blue in 1% borax. No grain counts were made; tissues were scored as unlabelled or weakly, moderately, or strongly labelled.

Results

Nuclear DNA synthesis

The development of *Asplanchna* has been described by Lechner (1966), Nachtwey (1925) and others. Complete embryonic development from mature egg to birth requires about 20 hours at 23°. During approximately the first five to six hours, all cells are undergoing rapid mitosis and cell division, with a cell generation time of 15 to 30 minutes. In this early, "mitotic phase" of development, approximately ten cleavages are completed. Few if any nuclei have prominent nucleoli. When embryos of these stages are exposed to H^3-thymidine for 30 minutes or more *in vitro,* all nuclei are heavily labelled as expected (Fig. 1). Preliminary studies indicate an S period of probably less than six minutes duration in the first two cell generations. Nuclei in the S period are labelled when embryos are exposed to H^3-thymidine for as few as 30 seconds.

Mitosis ceases at different times in different tissue anlagen, ranging from about five to seven hours. Nucleoli first appear at this time. Tissues with nucleoli do not appear to contain mitotic figures, suggesting a close correlation between the initial formation of nucleoli and the cessation of mitosis. Moreover, cells with nucleoli are only rarely labelled after short (less than 30 minutes) exposures to H^3-thymidine, suggesting a further correlation between the end of mitosis, nucleolar formation and the end of DNA synthesis. However, our experiments cannot exclude the possibility that some DNA synthesis occurs shortly after the last

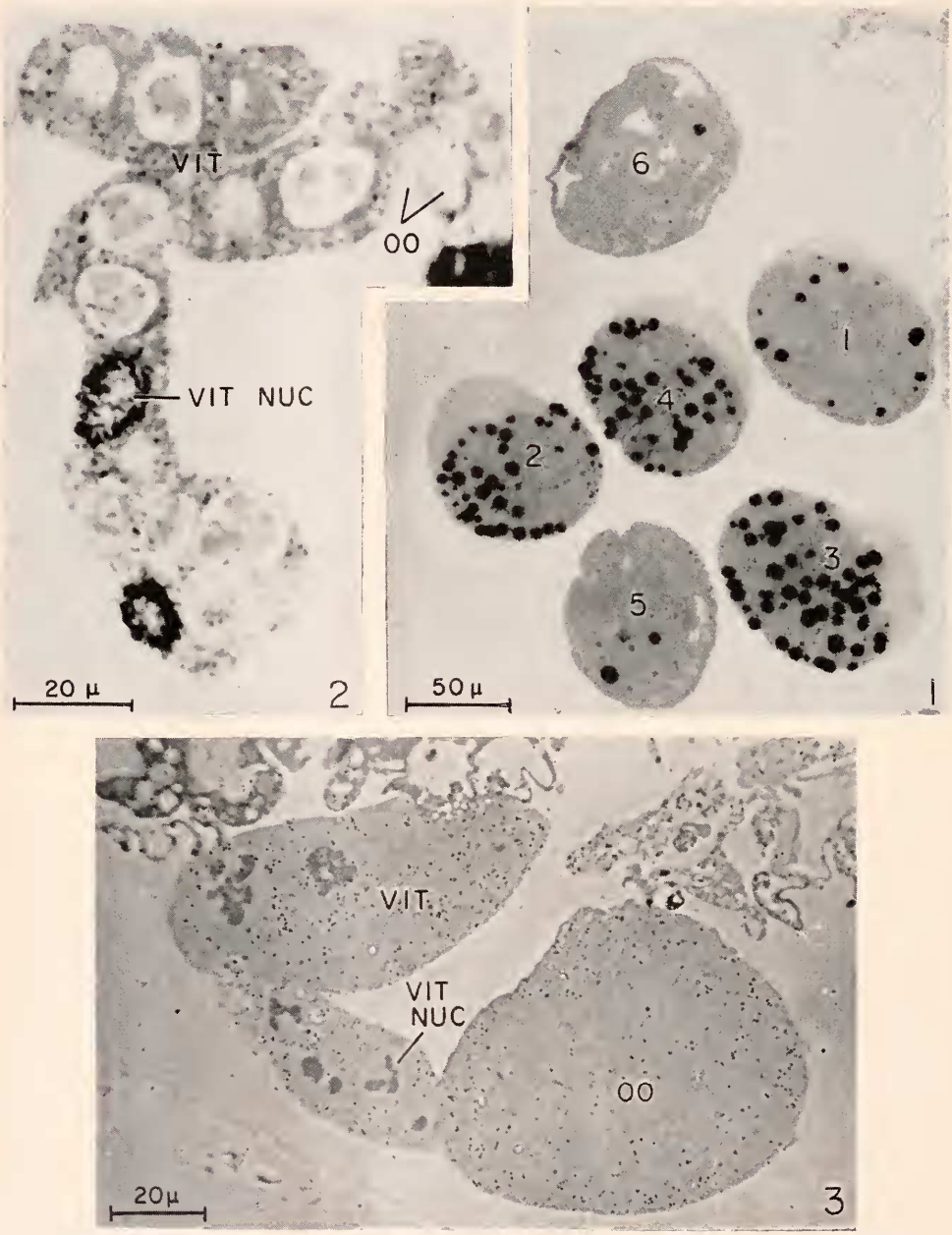

FIGURE 1. *Asplanchna* embryos labelled *in vitro* with H^3-thymidine for 1.5 hours; OsO_4 fixation, 0.5-micron methacrylate section, autoradiograph exposed 21 days. In mitotic phase embryos (1–3), all nuclei are heavily labelled; in transition embryo (4), some nuclei are not labelled, while in post-mitotic embryos (5 and 6), only a few vitellarium nuclei are labelled.

FIGURE 2. Vitellarium of mature female labelled *in vivo* for 4½ hours; glutaraldehyde and OsO_4 fixation, 2-micron methacrylate section, exposed 14 days. Immature oocytes (OO) are

mitosis, or that nucleoli are formed in some cells shortly before DNA synthesis and mitosis are completed.

In "post-mitotic" embryos and adults, most nuclei have large nucleoli, and no nuclei undergo mitosis. In these older animals, only the nuclei of the vitellarium incorporate H^3-thymidine into DNA (Figs. 1, 2). That this label is indeed in high-molecular weight DNA is indicated by several tests. The label is removed completely or nearly so from paraffin sections or squashes by treatment with DNase but not by the control buffer, ribonuclease, hot 1 *N* HCl, cold 5% TCA, or trypsin (Figs. 4, 5). Adult animals incorporate H^3-thymidine into the vitellarium nuclei at approximately the same rate in the presence of unlabelled uridine at 10 or 100 times the concentration of the thymidine. Finally, identical labelling patterns appear following the use of H^3-thymidine from two different sources and after a variety of different histological procedures.

The labelling of the vitellarium nuclei is highly variable in intensity, making quantitative data almost impossible to obtain. In early post-mitotic embryos, most or all vitellarium nuclei are labelled very heavily after even brief exposures of one hour or less *in vitro* or *in utero*. In contrast, single exposures of nearly mature embryos or of adults result in the labelling of from none to all of the nuclei in a given vitellarium. This variability is found even among animals labelled in the same experiment, under identical conditions. Moreover, the intensity of labelling varies from weak to strong among the different nuclei in a single vitellarium. There is some indication that the frequency of labelled nuclei decreases with age in mature females. The observed variability of labelling could be explained by either of two hypotheses: (1) a variable number of nuclei in adult vitellaria never synthesize DNA, or (2) all vitellarium nuclei carry out DNA synthesis in the adult, but at different times. One experiment argues in favor of the latter hypothesis. Newborn females were collected and exposed to H^3-thymidine in four successive pulses, of from 1 to $4\frac{1}{3}$ hours each, at ages zero, 18, 25, and 43 hours. Each pulse was followed by a chase exposure to unlabelled thymidine at 100 times the concentration of the H^3-thymidine. This regime resulted in uniform heavy labelling of all vitellarium nuclei in all females.

We have performed several chase experiments to test the metabolic stability of the labelled DNA in the vitellarium nuclei. Labelled females have been reared in the presence of a 100-fold concentration of cold thymidine for up to 47 hours (about one-half of the life span) without any noticeable decrease in the intensity of the nuclear label or in the number of labelled nuclei. Such experiments indicate that most or all of the DNA synthesized in adult vitellarium nuclei is metabolically stable, at least in young and middle-aged females.

Cytoplasmic DNA synthesis

Animals exposed to H^3-thymidine frequently show incorporation of the radioactive precursor in the cytoplasm (Fig. 3). This cytoplasmic label is largely or

not labelled. Two vitellarium nuclei (VIT NUC) are heavily labelled; the remaining nuclei and the cytoplasm are lightly labelled in the vitellarium (VIT).

FIGURE 3. Adult vitellarium (VIT) with attached, nearly-mature oocyte (OO) labelled as described in Figure 1. Note heavy cytoplasmic label; a few vitellarium nuclei (VIT NUC) are lightly labelled.

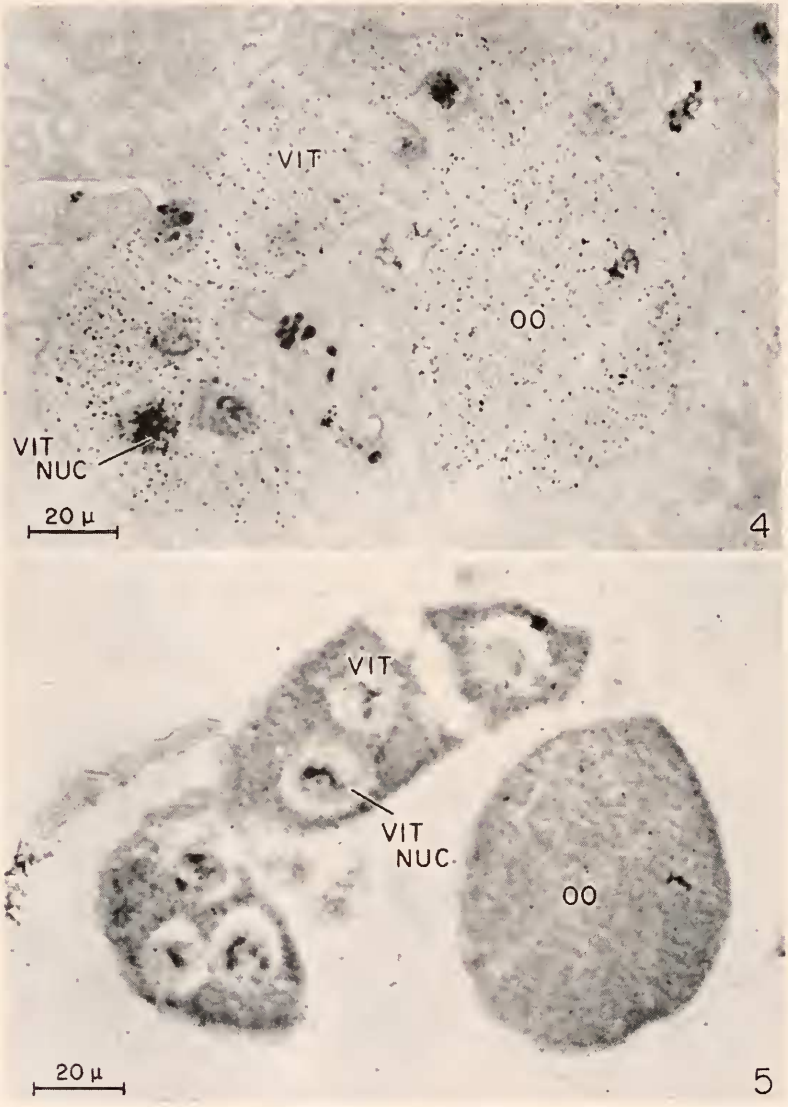

FIGURE 4. Adult vitellarium (VIT) and nearly-mature oocyte (OO) labelled *in vitro* for 30 minutes, fixed in 3:1 ethanol : acetic acid, and embedded in paraffin; the four-micron section was treated with ribonuclease at 0.3 mg./ml. for six hours. Autoradiograph exposed seven days; stained with Harris hematoxylin. Note heavy label over cytoplasm and in some nuclei (VIT NUC); almost complete absence of cytoplasmic basophilia indicates extraction of RNA.

FIGURE 5. Section adjacent to the one in Figure 4, treated with deoxyribonuclease at 0.3 mg./ml. for six hours. Note that cytoplasmic basophilia is retained, but most label has been removed.

entirely in high-molecular weight DNA, as indicated by the same criteria used for the nuclear label (Figs. 4, 5). As a general rule, cytoplasmic labelling is limited to the vitellaria of adults and nearly-mature embryos. However, in some experiments oocytes (Figs. 3, 4) and embryos in cleavage stages are also labelled. As was the case with the vitellarium nuclei, the labelling of cytoplasmic DNA is variable in intensity. When vitellaria are exposed to H^3-thymidine *in vitro* for periods of from 20 minutes to two hours, less than half of the vitellaria show detectable label in a given experiment. Exposure of adult females to H^3-thymidine for from 2 to 23 hours in saline solution results in the labelling of from none to all of the vitellaria in different experiments, with no apparent correlation with length of exposure. Exposure of adult females to H^3-thymidine in the presence of paramecia results in uniform heavy labelling of the cytoplasm, but much of the label is removed by RNase and trypsin. Because of technical difficulties we have not yet been able to obtain any conclusive information about the stability of the cytoplasmic DNA.

It is conceivable that the labelled cytoplasmic DNA is derived in part or entirely from labelled nuclear DNA. It seems more likely, however, that this DNA is synthesized *in situ*. Some vitellaria have heavily labelled nuclei but no detectable cytoplasmic label. Other vitellaria in the same experiment may show cytoplasmic label but little or no nuclear label. For these same reasons, incidentally, a transfer of labelled DNA from the cytoplasm to the nucleus is also unlikely. We are currently using electron microscope autoradiography, as well as light microscope autoradiography under conditions which allow visualization of vitellarium mitochondria, to determine the precise location of the labelled cytoplasmic DNA. Our preliminary results indicate that most or all of the labelled DNA is in mitochondria.

Discussion

Autoradiographic studies such as this one do not distinguish between the replication of DNA molecules, *de novo* synthesis of new molecules without a template, repair of molecules, or the terminal addition of nucleotides onto pre-existing molecules. Nor do they distinguish between DNA which is a functional part of the genome and any other DNA which might be found in the nucleus or cytoplasm. However, in the absence of evidence for more exotic processes, it is reasonable to assume that the incorporation of H^3-thymidine into nuclear DNA represents replication of the genome entirely or in part, even in the non-mitotic vitellarium nuclei of the adult. Indeed, polytenization and polyploidization are common in nurse cells, especially in insects (*cf.* King, 1964; Raven, 1961). In *Asplanchna*, the nurse cells are probably becoming polyploid rather than polytene, as no trace of polytene chromosomes has been seen with either the light or the electron microscope.

A form of limited polyploidy has been reported in amphibian oocytes, in which those DNA cistrons which code for ribosomal RNA are replicated many-fold and the replicas released from the chromosomes to form many independent nucleoli in the nuclear sap (Izawa *et al.*, 1963; Miller, 1964).

In *Asplanchna* there is only one nucleolus in each vitellarium nucleus, and much of the labelled DNA is not closely associated with the nucleolus. Thus if limited polyploidization is involved, it is probably not limited to the nucleolar organizer or ribosomal RNA cistrons. It is conceivable that extra copies of the vitellarium

genome, or of selected regions thereof, are required to support rapid synthesis of the cytoplasm which is supplied to the maturing oocyte.

During the post-mitotic phase of rotifer development, cell membranes break down and disappear completely in most tissues, including the vitellarium. This organ is thus a true syncytium during the period of variable nuclear DNA synthesis. Moreover, it is connected to both immature and maturing oocytes by broad cytoplasmic channels. The variability in time and rate of DNA synthesis in the vitellarium nuclei, and the complete absence of DNA synthesis in the oocytes, thus indicate that the cytoplasm of the reproductive organs does not exert control over nuclear DNA synthesis. Similar phenomena have been found in the *Drosophila* egg chamber (Jacob and Sirlin, 1959).

The rotifers may now be added to the growing list of organisms whose mitochondria contain DNA (see Pikó *et al.*, 1967, for references). Cytoplasmic incorporation of H^3-thymidine into DNA has also been reported in another aschelminth, the nematode, *Caenorhabditis briggsae* (Nonnenmacher-Godet and Dougherty, 1964); the precise location of this DNA has, however, not been determined. It appears that DNA can now be considered to be a universal constituent of mitochondria. We are now conducting experiments designed to test the heredity of this DNA during parthenogenetic reproduction.

The authors are grateful to Dr. Daniel Mazia for advice during this project, and to Dr. W. M. Laetsch for the use of his photomicrographic equipment.

Summary

1. DNA synthesis has been studied in adult and embryonic tissues of the rotifer *Asplanchna brightwelli*, using autoradiography with tritiated thymidine.

2. Mitosis and mitotic DNA synthesis cease, and nucleoli are formed, approximately simultaneously at the end of the mitotic phase of embryonic development.

3. In the post-mitotic phase of development and in adult females, the large nuclei of the vitellarium (a nurse cell-like organ) incorporate H^3-thymidine into metabolically-stable DNA, thus presumably becoming polyploid.

4. The DNA synthesis in the different nuclei of the syncytial vitellarium is not synchronous, showing that the cytoplasm does not control nuclear DNA synthesis.

5. Cytoplasmic DNA synthesis also occurs in embryos and in adult vitellaria. Most or all of the cytoplasmic DNA appears to be in the mitochondria.

LITERATURE CITED

Birky, C. W., Jr., 1964. Studies on the physiology and genetics of the rotifer, *Asplanchna*. I. Methods and physiology. *J. Exp. Zool.*, **155**: 273–292.

Birky, C. W., Jr., 1967a. Studies on the physiology and genetics of the rotifer, *Asplanchna*. III. Results of outcrossing, selfing, and selection. *J. Exp. Zool.*, **164**: 1–11.

Birky, C. W., Jr., 1967b. Rotifers. *In*: Experimental Methods in Developmental Biology (F. Wilt and N. Wessells, ed.). Thomas Y. Crowell Company, N. Y. (in press)

Birky, C. W., Jr., and B. Field, 1966. Nuclear number in the rotifer *Asplanchna*: intraclonal variation and environmental control. *Science*, **151**: 585–587.

Caro, L. G., 1964. High-resolution autoradiography. *In*: Methods in Cell Physiology (D. M. Prescott, ed.), **1**: 327–363.

Dryl, S., 1959. Antigenic transformation in *Paramecium aurelia* after homologous antiserum treatment during autogamy and conjugation. *J. Protozool.*, **6** (suppl.): 25.

Gilbert, J. J., 1963. Mictic female production in the rotifer *Brachionus calyciflorus*. *J. Exp. Zool.*, **153**: 113–123.

Izawa, M., V. G. Allfrey and A. E. Mirsky, 1963. The relationship between RNA synthesis and loop structure in lampbrush chromosomes. *Proc. Nat. Acad. Sci.*, **49**: 544–551.

Jacob, J., and J. L. Sirlin, 1959. Cell function in the ovary of *Drosophila melanogaster*. I. DNA classes in the nurse cell as determined by autoradiography. *Chromosoma*, **10**: 210–228.

King, R. C., 1964. Studies on early stages of insect oogenesis. *In*: Insect Reproduction. Symp. Royal Entomological Soc. London No. 2: 13–25.

Lechner, M., 1966. Untersuchungen zur Embryonalentwicklung des Rädertieres *Asplanchna girodi* de Guerne. *Arch. f. Entw.*, **157**: 117–173.

Miller, O. L., 1964. Extrachromosomal nucleolar DNA in amphibian oocytes. *J. Cell Biol.*, **23**: 60A.

Nachtwey, R., 1925. Untersuchungen über die Keimbahn, Organogenese und Anatomie von *Asplanchna priodonta* Gosse. *Zeit. wiss. Zool.*, **126**: 239–492.

Nonnenmacher-Godet, J., and E. C. Dougherty, 1964. Incorporation of tritiated thymidine in the cells of *Caenorhabditis briggsae* (Nematoda) reared in axenic culture. *J. Cell Biol.*, **22**: 281–290.

Piko, L., A. Tyler and J. Vinograd, 1967. Amount, location, priming capacity, circularity and other properties of cytoplasmic DNA in sea urchin eggs. *Biol. Bull.*, **132**: 68–90.

Raven, C. P., 1961. Oogenesis. Pergamon Press, N. Y.